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Effect of Coadministration of Vancomycin and BMP-2 on Cocultured *Staphylococcus aureus* and W-20-17 Mouse Bone Marrow Stromal Cells *In Vitro*

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In this study, we aimed to establish an *in vitro* bacterium/bone cell coculture model system and to use this model for dose dependence studies of dual administration of antibiotics and growth factors *in vitro*. We examined the effect of single or dual administration of the antibiotic vancomycin (VAN) at 0 to 16 µg/ml and bone morphogenetic protein-2 (BMP-2) at 0 or 100 ng/ml on both methicillin-sensitive *Staphylococcus aureus* and mouse bone marrow stromal cells (W-20-17) under both mono- and coculture conditions. Cell metabolic activity, Live/Dead staining, double-stranded DNA (dsDNA) amounts, and alkaline phosphatase activity were measured to assess cell viability, proliferation, and differentiation. An interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kit was used to test the bone cell inflammation response in the presence of bacteria. Our results suggest that, when delivered together in coculture, VAN and BMP-2 maintain their primary functions as an antibiotic and a growth factor, respectively. Most interestingly, this dual-delivery type of approach has shown itself to be effective at lower concentrations of VAN than those required for an approach relying strictly on the antibiotic. It may be that BMP-2 enhances cell proliferation and differentiation before the cells become infected. In coculture, a dosage of VAN higher than that used for treatment in monoculture may be necessary to effectively inhibit growth of *Staphylococcus aureus*. This could mean that the coculture environment may be limiting the efficacy of VAN, possibly by way of bacterial invasion of the bone cells. This report of a coculture study demonstrates a potential beneficial effect of the coadministration of antibiotics and growth factors compared to treatment with antibiotic alone.

The regeneration of contaminated bony tissue defects poses itself as one of the major areas of concern in the field of bone tissue engineering, especially with regard to war traumas in extremities, where infection of open type III fractures is all but certain (10, 33). These bacterial infections complicate the already strenuous repair process, often resulting in slower union rates or even amputation (7, 22). At the present, standard clinical care is a two-phase process. First, antibiotics are administered to stop any ongoing infection of the bony tissue if present. Second, a bone graft is implanted, and bone growth factors are delivered to facilitate the regrowth of natural bone tissue by using the patient's resident osteoblasts. While this treatment protocol is designed to prevent contamination and promote bone growth, recurrent infection following implantation of the graft continues to be a major barrier to generating consistently positive outcomes (2, 19, 29, 48).

One suggested approach to combating this issue is to use a specialized synthetic bone graft material capable of delivering both antibiotics and growth factors locally after implantation (9, 16, 25, 31, 46, 47, 50). While antibiotic integration into bone grafts has been proven successful, less work has been done to deliver an antibiotic and a growth factor together (either in series or parallel) (24, 25). As a result of limiting the need for the antibiotic to travel systemically, there are higher antibiotic levels at the site of the wound and systemically safe levels, thus paving the way for resident osteoblasts to penetrate into the graft well before the offending bacteria are able to reach high confluence and form biofilms (38). Moreover, combined treatment with bone growth factors such as bone morphogenetic protein-2 (BMP-2) can accelerate the healing process in a simple, streamlined delivery approach (14,

20, 23, 28, 34, 37). With the emergence of these complex dual-purpose grafts, the need for a flexible *in vitro* test system highly representative of *in vivo* conditions is of paramount importance in helping to smooth the natural transition into animal studies. Such a model system would allow more realistic assessment of different clinical treatment options in a rapid, cost-efficient, and safe manner, especially with regard to testing possibly host-toxic therapies.

Here, we aimed to establish an *in vitro* bacterium/bone cell coculture model system and to use this model for dose dependence studies of the dual administration of antibiotics and growth factors *in vitro*. We examined the interactions between our two tested model cell lines (W-20-17 mouse bone marrow stromal cells [mBMSCs] and methicillin-sensitive *S. aureus* ATCC 6538) as well as their responses to various treatments with vancomycin (VAN) at 0 to 16 µg/ml and BMP-2 at 0 or 100 ng/ml both in mono- and coculture (13). *S. aureus* is a highly infectious Gram-positive bacterium known for its ability to internalize itself within mammalian cells and is the predominant cause of bone graft failures (2, 3, 8, 9, 11, 12, 15, 19, 29, 30, 32, 36, 44, 48). W-20-17 is derived from mouse bone marrow stromal cells and has been used as an ASTM (F2131) standard test for *in vitro* biological activity of

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BMP-2. Previous studies showed that BMP-2 significantly stimulated alkaline phosphatase (ALP) activity in W-20-17 in a dose-dependent manner (26). With so many antibiotics available for treatment of infections by Gram-positive bacterial strains such as our chosen methicillin-sensitive *S. aureus* (MSSA) strain, vancomycin presents itself as one of the most aggressive antibiotics available for clinical use and has been proven to work very well against methicillin-resistant *S. aureus* (MRSA) while still being effective against MSSA (21, 38, 39, 41, 43). While toxicity of the drug is often considered a deterrent for its use, vancomycin is only toxic at levels well above the MIC for nonresistant *S. aureus* (21, 43). Regardless, we also assessed whether or not concentrations of vancomycin well above our suspected working levels (up to 200 µg/ml) would be toxic to our W-20-17 cells. Because of the many complications involved in performing cocultures of this nature, we utilized a modified version of a previously created system in order to establish our model (the details of which are explained below) (6, 13). Note that the design of our system is fairly modular, allowing the substitution of different cell lines and substances in order to meet the demands of multiple experimental conditions.

We hypothesized that BMP-2 and VAN would maintain their respective primary functions on their target cell lines when delivered together in our coculture system. To test this, cell viability, proliferation, and differentiation under an array of conditions and across a number of time points were measured. Our findings show that even when delivered together in coculture, VAN and BMP-2 do not lose their functionality as an antibiotic and a growth factor, respectively. Moreover, some evidence suggests that the addition of BMP-2 can reduce the amount of VAN necessary to inhibit bacterial growth and thus allow more rapid bone cell proliferation and differentiation.

MATERIALS AND METHODS

Culture of bacteria. A clinical strain of *S. aureus* (ATCC 6538) was propagated according to the guidelines provided by the vendor. Briefly, cells were grown in 200 ml of tryptic soy broth (TSB) in a 1-liter Erlenmeyer flask and incubated at 37°C in a humidified incubator. Once cells reached an optical density at 600 nm (OD₆₀₀) of ~0.5, they were centrifuged (10 min at 4,300 × g and 4°C) and resuspended in a 20% glycerol solution. Aliquots of this suspension were then frozen in liquid nitrogen and stored at -80°C until needed for culture.

Culture of mouse bone cells. W-20-17 cells (ATCC) were propagated according to the guidelines provided by the vendor. Briefly, cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), a 1% antibiotic/antimycotic mixture, 5 ml of L-glutamine (200 mM), and sodium pyruvate. This cell line is an ASTM standard to evaluate activity of BMP-2 *in vitro*. The cells were cultured in an incubator supplied with 5% CO₂ at 37°C. Medium was replaced every 3 days. To prevent oversaturation of the flask, cultures were periodically subjected to passage prior to the cells reaching high confluence.

Preparation of VAN and BMP-2 treatments. Vancomycin treatments were prepared by dissolving dry vancomycin hydrochloride (Acros Organics) in phosphate-buffered saline (PBS). Serial dilutions were performed to create stocks with concentrations of 160, 80, 40, 20, and 10 µg of VAN/ml (1, 5, 17, 21, 40, 43). These stocks were then run through a sterile filter and refrigerated until use. BMP-2 stocks (R&D Inc.) were similarly suspended in PBS and stored at -20°C until use.

Experimental design. For monocultures, *S. aureus* bacteria were grown in 24-well plates. Appropriate volumes of TSB, VAN, BMP-2, and phosphate-buffered saline (PBS) were added to each well such that the

final volume was 500 µl (1,000 µl for cultures used in ALP and double-stranded DNA [dsDNA] assays). Cells from thawed frozen stock were seeded to a final concentration of 10⁵ CFU/ml and incubated at 37°C in a humidified incubator (5% CO₂). Tested final concentrations of VAN were 0, 1, 2, 4, 8, and 16 µg/ml, while tested concentrations of BMP-2 were 0 and 100 ng/ml. W-20-17 cells were also grown in monoculture in 24-well plates. Cells were seeded to a final concentration of 15,000 cells per well in DMEM and incubated at 37°C in a humidified incubator (5% CO₂). Tested final concentrations of VAN were 0, 1, 2, 4, 8, and 16 µg/ml, while tested concentrations of BMP-2 were 0 and 100 ng/ml. For treatments requiring either zero VAN or zero BMP-2, the appropriate volume of PBS was added instead. For culture growth for longer times, culture medium and treatments were replaced with fresh medium and the appropriate amounts of each VAN or BMP-2 treatment every 3 days. All concentrations were kept constant throughout the culture period.

Bacterial cells used for coculture were taken from a growing liquid culture. A 125-ml flask containing 10 ml of TSB was inoculated with *S. aureus* bacteria and allowed to grow overnight in a humidified incubator (37°C, 5% CO₂), with shaking at 200 rpm. The following day, the culture was centrifuged (10 min at 4,300 × g and 4°C) and the pellet was washed with 10 ml of Hank's balanced salt solution (HBSS) and resuspended in 10 ml of DMEM-p (DMEM, 10% FBS, 0% penicillin, 5 ml of L-glutamine [200 mM], and sodium pyruvate). Cocultures of *S. aureus* and W-20-17 cells were performed through a series of incubations, washes, and medium changes. First, W-20-17 cells were cultured overnight as described previously. After incubation, culture wells were aspirated and rinsed twice with 500 µl of HBSS before replacement of the medium with 480 µl of DMEM-p. Next, the mouse cell cultures were infected with 20 µl of *S. aureus* suspension for an approximate final concentration of 10⁷ CFU/ml, a concentration higher than that used for monocultures in order to aid in the differentiation of treatment groups by using a more stringent set of conditions. This coculture was then incubated for 45 min at 37°C. Following the 45-min infection period, cell culture wells were aspirated and washed twice with 500 µl of HBSS to remove as much of the extracellular bacteria as possible and incubated at 37°C in 400 µl of DMEM-p and 100 µl of a predetermined treatment combination of VAN (0, 1, 2, 4, 8, or 16 µg/ml) and BMP-2 (0 or 100 ng/ml).

Measurement of metabolic activity. Metabolic activity was measured using Promega CellTiter96 Aqueous One solution (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) as directed by the supplier. At predetermined time points, the number of viable cells was determined quantitatively according to the manufacturer's instructions. For monocultures, the metabolic activity of *S. aureus* or W-20-17 cells was tested separately (in TSB or DMEM, respectively). For cocultures, the metabolic activity of W-20-17 cells was evaluated by culturing a *S. aureus* monoculture (10⁷ CFU/ml) in parallel with the coculture (using the same treatments and under the same conditions) and subtracting the monoculture background from the coculture measurement. To remove as much of the residual bacteria as possible, culture medium was aspirated, washed twice with 500 µl of HBSS, supplied with 400 µl of fresh DMEM-p and 100 µl of the treatment, and incubated for 30 min before the reagent was added. Assays began with 100 µl of the reagent being added to each well of the 24-well plate. Cultures were then placed in a humidified incubator at 37°C for 1 h. Finally, 100 µl of each well was transferred to a 96-well assay plate and measured at 490 nm in a microplate reader (TECAN Infinite F50).

Staining and visualization of cells. Qualitative analysis of cell cultures was accomplished using Invitrogen Live/Dead (L/D) cell viability kits as directed by the supplier. Bacterial monocultures were visualized using a Live/Dead BacLight Bacterial Viability kit, whereas W-20-17 monocultures and cocultures were visualized using a Live/Dead Mammalian Cell Viability kit. Photomicrographs of cells were documented using a microscope (Nikon Eclipse TE-2000-U) and processed using MetaVue software (Nikon MetaVue) after 1, 2, and 3 days of W-20-17 monoculture, 3 days of *S. aureus* monoculture, and 1, 3, and 7 days of coculture.

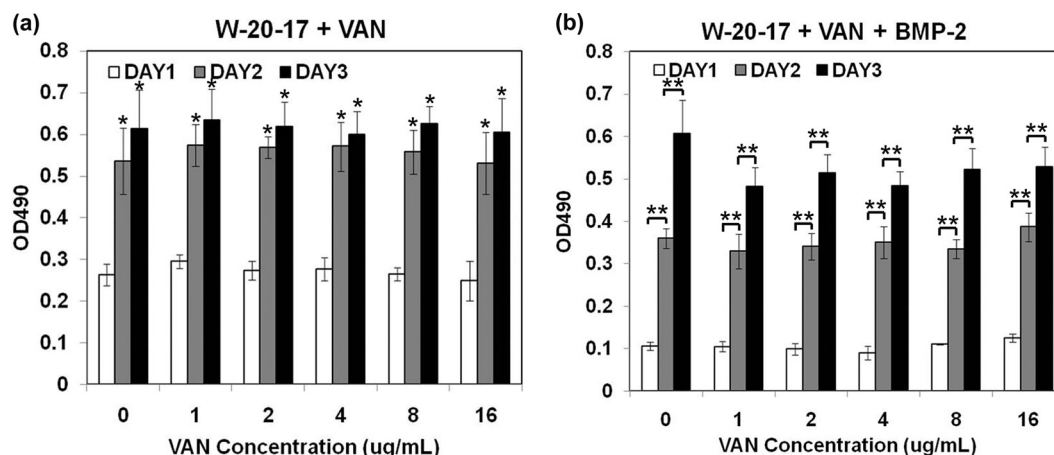


FIG 1 Measure of metabolic activity of W-20-17 treated with 0 to 16 µg/ml VAN in combination with (a) 0 ng/ml BMP-2 or (b) 100 ng/ml BMP-2 over 3 days using an MTS colorimetric assay. Higher OD₄₉₀ values indicate greater metabolic activity. The initial cell density was 15,000 cells per well for 3 days. The absorbance is expressed as a measure of the cell viability via cell culture media for 3 days of incubation. Each value represents the mean ± SD (*n* = 3). *, statistically significant difference compared with 1 day of culture (*P* < 0.05). **, statistically significant difference between different time points (*P* < 0.05).

Preparation of cell culture lysate for ALP and dsDNA. At the designated time, the medium was removed from the cell culture. The cell layers of W-20-17 were washed twice with PBS (pH 7.4) and then lysed with 1 ml of 0.2% Triton X-100 by three freeze-thaw cycles, which consisted of freezing at −80°C for 30 min immediately followed by thawing at 37°C for 15 min. The cell lysates were used to determine ALP activity and double-stranded DNA levels.

Preparation of bacterial cell culture lysate for ALP and dsDNA. At the designated time, the samples in each well were mixed and transferred to microcentrifuge tubes and centrifuged for 15 min at 10,000 × *g* and 4°C. The supernatant was removed, and the pellet was then resuspended in 1,000 µl of 0.2% Triton X-100. Lysis was achieved by performing four freeze-thaw cycles, which consisted of freezing at −80°C for 30 min immediately followed by thawing at 37°C for 15 min. The cell lysates were used to determine ALP activity and double-stranded DNA levels.

Measurement of ALP activity. The cell lysates were assayed for the presence of ALP, an important marker for determining osteoblast phenotype. Aliquots (50 µl) of the cell lysates were sampled and added to 50 µl of working reagent in a 96-well assay plate. The working reagent contained equal parts (1:1:1) of 1.5 M 2-amino-2-methyl-1-propanol (Sigma), 20 mM *p*-nitrophenyl phosphate (Sigma), and 1 mM magnesium chloride. The samples then were incubated for 1 h at 37°C. After incubation, the reaction was stopped with 100 µl of 1 N sodium hydroxide on ice. ALP activity was determined from the absorbance using a standard curve prepared from *p*-nitrophenol stock standard (Sigma). The absorbance was measured at 405 nm using a microplate reader (Bio-Rad model 680). The ALP activity of cells was then calculated by normalizing to double-stranded DNA (dsDNA). ALP activity was expressed as nanomoles per nanogram.

Measurement of dsDNA amount. The cell lysates were examined for double-stranded DNA to estimate cell numbers. Aliquots (50 µl) of the cell lysates were added in a 96-well assay plate. A 50-µl volume of a 1:200 dilution of PicoGreen (Quant-iT PicoGreen assay kit; Invitrogen) was added to each well and incubated for 5 min in the dark. The assay plate was read (485-nm excitation and 530-nm emission) using a BioTek FLx800 plate reader. The double-stranded DNA content was calculated using a standard curve made using a provided dsDNA standard sample. Amounts of dsDNA were measured using a PicoGreen fluorescence assay to standardize ALP production measurements.

Measurement of IL-6 response. Interleukin-6 (IL-6) production by W-20-17 in coculture was measured using an Invitrogen IL-6 enzyme-linked immunosorbent assay (ELISA) kit with minor modifications to the

sampling protocol provided by the supplier. Samples tested were prepared from the supernatant of each culture. Briefly, *S. aureus*/W-20-17 cocultures and W-20-17 monocultures were performed as described previously. After 3 days of incubation, the medium from each well was transferred to individual microcentrifuge tubes and centrifuged for 5 min at 300 × *g* and 4°C. The resulting supernatant was then stored at −20°C until measurement using the ELISA kit. The assay was performed as written in the kit's instructions. Briefly, the collected samples were added to the assay well plate in duplicate. The prescribed series of reagent additions, washes, and incubations were performed as instructed. Following the addition of stop solution, absorbance was measured using a Tecan Infinite F50 microplate reader set to 450 nm.

Statistical analysis. All data are presented as means ± standard deviations. For comparing two groups of data, a Student *t* test was performed. For comparing multiple groups of data, one-way analysis of variance (ANOVA) was performed followed by Tukey's test. The differences in data for groups and experimental time points were considered statistically significant when *P* < 0.05.

RESULTS

Mouse cell metabolic activity in monoculture. MTS assays were performed to measure cell metabolic activity at various concentrations of antibiotic VAN (between 0 and 16 µg/ml) and BMP-2 (either 0 or 100 ng/ml) over a 3-day period. The results were used to determine effectiveness at the bacterial concentration tested in coculture (Fig. 1). W-20-17 cells showed significantly higher metabolic activity at day 3 than at day 1 (*P* < 0.05), indicating that the cells were able to grow and proliferate in the presence of VAN. Our results indicate that VAN had little to no measurable effect on overall cell metabolic activity regardless of concentration, BMP-2 presence, or culture duration. Worth noting is that all groups treated with BMP-2 (Fig. 1b) showed lower metabolic activity relative to their BMP-2-free counterparts (Fig. 1a). This suggests that BMP-2 may reduce the overall metabolic activity of W-20-17 cell cultures (possibly by reducing cell proliferation in favor of cell differentiation). Live/Dead fluorescence staining also showed that in all treatment groups, W-20-17 cells were viable and maintained their normative spindle-like shapes (Fig. 2). Furthermore, visual assessment indicates an increase in cell proliferation over the 3 days of culture across all treatments of VAN and BMP-2.

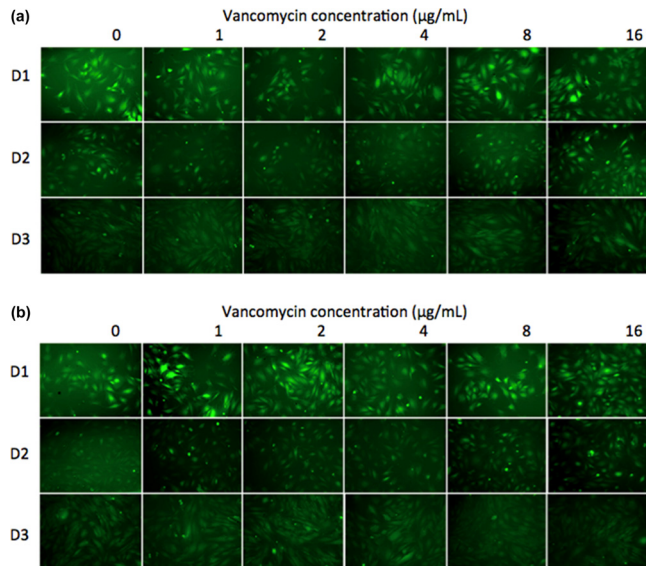


FIG 2 Live/Dead fluorescence staining of W-20-17 treated with 0 to 16 µg/ml VAN and either (a) 0 ng/ml BMP-2 or (b) 100 ng/ml BMP-2 in monoculture in DMEM. Photos were taken at $\times 100$ magnification after up to 3 days of culture (D1, day 1; D2, day 2; D3, day 3). Green indicates live bone cells, and red indicates dead cells.

Mouse cell osteoblastic differentiation in monoculture. ALP assays were performed to measure osteogenic activity of W-20-17 cells at various concentrations of antibiotic VAN (between 0 and 16 µg/ml) and BMP-2 (either 0 or 100 ng/ml) over a 7-day period. Our results indicate that VAN had no clear effect on ALP-specific activity (ALP normalized to dsDNA per unit) regardless of concentration, BMP-2 presence, or day (Fig. 3). The results suggest that BMP-2 was able to maintain its ability to increase overall ALP activity at all concentrations of VAN. dsDNA levels were measured in order to normalize ALP activity. Results indicate little overall difference in dsDNA levels at day 7, but there was a consistent trend of reduced dsDNA levels at day 5 in the BMP-2-treated groups relative to their BMP-2-free counterparts. Regardless, a statistically significant difference existed between the 0

ng/ml and 100 ng/ml BMP-2 groups in terms of ALP-specific activity.

Bacterial metabolic activity in monoculture. MTS assays were performed to measure cell metabolic activity at various concentrations of VAN antibiotic (between 0 and 16 µg/ml) and BMP-2 (either 0 or 100 ng/ml) over a 24-h period (Fig. 4). Our results indicate that at every measurement time, all treatment groups with VAN at ≥ 1 µg/ml showed highly suppressed *S. aureus* growth (for all intents and purposes, *S. aureus* metabolic activity was practically zero in these groups). The treatment group with no VAN showed dramatic increases in metabolic activity by 6 h and immeasurably high levels by 24 h. Overall, our data suggest that even at concentrations of 1 µg/ml, VAN was effective at hindering the growth of our strain of *S. aureus* when seeded at 10^5 CFU/ml. However, because this result proved incapable of distinguishing the levels of efficacy of our treatments, the assays used for Live/Dead fluorescence staining of *S. aureus* as well as all coculture assays were seeded with bacteria at 10^7 CFU/ml. At this higher concentration, L/D staining also confirmed that *S. aureus* lost their viability after treatment with VAN at a concentration of at least 2 µg/ml regardless of the presence or absence of BMP-2 (Fig. 5). Visually, there was a decrease in bacterial cell proliferation within the 3 days of culture across all treatments performed with VAN.

Bacterial ALP production in monoculture. Bacterial ALP production was measured to assess whether or not the amount of ALP produced by the tested *S. aureus* strain was significant compared to W-20-17 cell results. Therefore, a high concentration of *S. aureus* cells (10^9 CFU) was cultured in 1 ml of DMEM-p in a shaking incubator for 3 h. ALP activity was measured as described previously. Results indicated that *S. aureus* strain ATCC 6538 produced about 0.045 nmol of ALP per ng of dsDNA. This is more than 10-fold less than the ALP-specific activity of W-20-17 cultured under similar conditions and is over 100-fold less than the ALP-specific activity of W-20-17 cultured with BMP-2. Despite this difference, the possibility cannot be ruled out that ALP production of *S. aureus* affects measurements taken from coculture. To suppress the possibly confounding variable of gross bacterial ALP production, ALP-specific activity (gross ALP data normal-

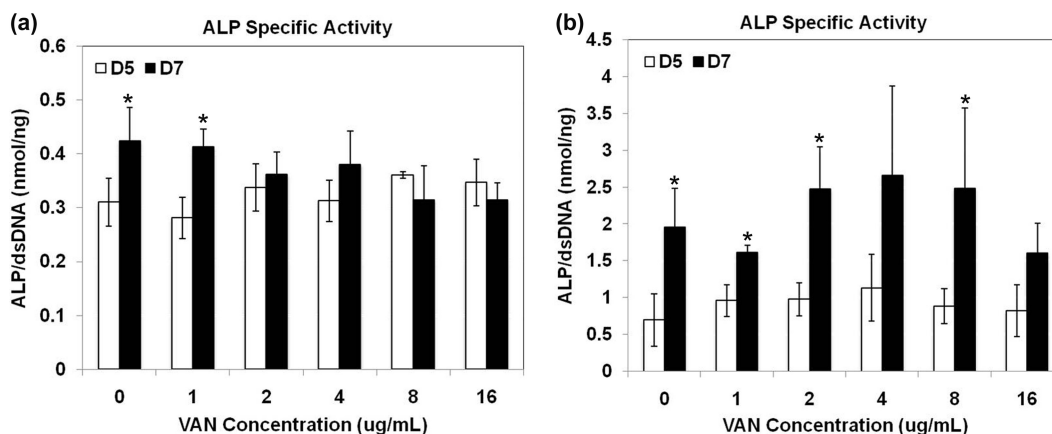


FIG 3 Measure of early osteoblastic differentiation of W-20-17 treated with 0 to 16 µg/ml VAN in combination with (a) 0 ng/ml BMP-2 or (b) 100 ng/ml BMP-2 in monoculture using an ALP assay. The ALP activity was determined at 5 and 7 days of culture and normalized for the dsDNA content. ALP activity is expressed as nanomoles per nanogram. Each value represents the mean \pm SD ($n = 3$). *, statistically significant difference compared with 5 days of culture ($P < 0.05$).

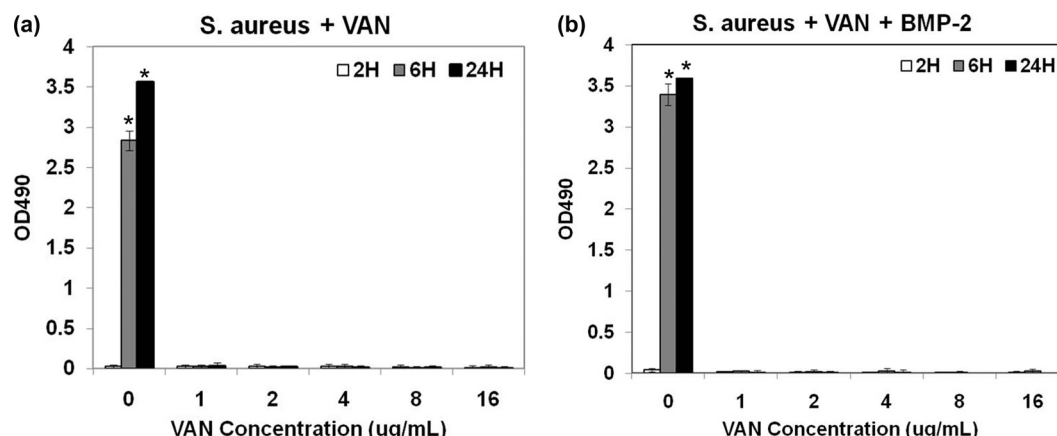


FIG 4 Measure of metabolic activity of *S. aureus* treated with 0 to 16 $\mu\text{g/ml}$ VAN in combination with (a) 0 ng/ml BMP-2 or (b) 100 ng/ml BMP-2 over 24 h using an MTS colorimetric assay. The initial concentration of *S. aureus* was 10^5 CFU/ml. Higher OD₄₉₀ values indicate greater metabolic activity. The absorbance is expressed as a measure of the cell viability via cell culture media for 24 h of incubation. Each value represents the mean \pm SD ($n = 3$). *, statistically significant difference compared with 2 h of culture ($P < 0.05$).

ized to dsDNA levels) was used for coculture assessment rather than raw ALP measurements.

Metabolic activity of mouse cells in coculture. MTS assays were performed to measure cell metabolic activity at various concentrations of VAN antibiotic (between 0 and 16 $\mu\text{g/ml}$) and BMP-2 (either 0 or 100 ng/ml) over a 7-day period (Fig. 6). L/D staining imaging was also performed to investigate viabilities of cells (Fig. 7). The results indicated that treatments with VAN at greater than 4 $\mu\text{g/ml}$ were effective at stopping bacterial growth in a coculture over a 7-day period. However, it was also found that treatment with VAN at between 1 and 2 $\mu\text{g/ml}$ represented the minimum concentration range required to effectively combat bacterial growth after 4 h in monoculture when bacteria were seeded at 10^7 CFU/ml (Fig. 5). Note that the MTS assay cannot distinguish between the metabolic activities of residual bacteria and those of growing W-20-17 cells when grown in coculture. Given the qualitative staining results, it is clear that the large increases in OD₄₉₀ values in groups treated with VAN at less than 8 $\mu\text{g/ml}$ can primarily be attributed to *S. aureus* growth. Groups treated with VAN at $\geq 8 \mu\text{g/ml}$ showed metabolic activity that was likely attributable to the W-20-17 cells. Note that treatment with VAN at 16 $\mu\text{g/ml}$ was sufficient for both concentrations of BMP-2 tested. However, a significant decrease in metabolic activity by day 7 was evident in groups treated with VAN at only 8 $\mu\text{g/ml}$ whereas there was an increase in activity in the comparable treatment group with BMP-2 at 100 ng/ml. This suggests that treatment with

8 $\mu\text{g/ml}$ VAN may not be sufficient to retain W-20-17 growth unless coupled with BMP-2 at 100 ng/ml. As shown in Fig. 7, the minimum VAN concentration that allowed W-20-17 growth increased at each time point regardless of BMP-2 treatment (red arrows). Staining results showed that with BMP-2 at both 0 and 100 ng/ml, 2 $\mu\text{g/ml}$ VAN was effective for 1 day, 4 $\mu\text{g/ml}$ for 3 days, and 8 $\mu\text{g/ml}$ for 7 days. Qualitatively, treatments with higher concentrations of VAN showed greater W-20-17 proliferation and reduced bacterial growth. When W-20-17 growth was evident, treatments with BMP-2 showed greater growth than those without.

Osteoblastic differentiation of mouse cells in coculture. ALP-specific activity was measured in order to assess the osteoblastic differentiation of W-20-17 cells in response to different vancomycin doses in a coculture environment over a 7-day period (Fig. 8). Treatments with VAN at 4 $\mu\text{g/ml}$ or a lower concentration were ineffective at inhibiting bacterial growth (as indicated by low or decreasing measurements of ALP-specific activity) regardless of the BMP-2 concentration. By day 7, ALP-specific activities showed a marked increase over day 3 activities in groups treated with VAN at 8 $\mu\text{g/ml}$ in combination with BMP-2 at 100 ng/ml but not in groups without BMP-2. This suggests that the combination of BMP-2 and VAN allows enhanced osteoblastic differentiation when cells are challenged with the parasitic *S. aureus* bacteria in coculture. ALP-specific activities in the coculture with VAN at 8 $\mu\text{g/ml}$ and 16 $\mu\text{g/ml}$ were similar to those seen with the monoculture. As expected, BMP-2-treated cultures that survived the 7-day period exhibited significantly greater ALP production than those without BMP-2, suggesting that BMP-2 maintains its effectiveness in coculture.

dsDNA amounts were primarily measured to assess ALP-specific activity. As a whole, though, dsDNA levels act as a general measure of cell proliferation. A progressive increase in dsDNA levels over the 7-day culture was evident, despite staining evidence suggesting the presence of very few W-20-17 cells in the cultures with low VAN concentrations. This was likely the result of bacterial dsDNA influencing measurements. To correct for this, we have reported only ALP-specific activity. Because we know that bacterial cells produce much lower concentrations of ALP than

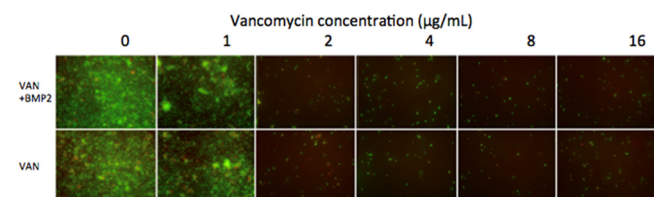


FIG 5 Live/Dead fluorescence staining of *S. aureus* treated with 0 to 16 $\mu\text{g/ml}$ VAN in monoculture and 0 or 100 ng/ml BMP-2 in TSB medium. Photos were taken at $\times 100$ magnification after 4 h of culture. Green indicates live bacterial cells, whereas red indicates dead cells. A visual decrease in cell viability over the 3 days of culture across all treatments of VAN is evident.

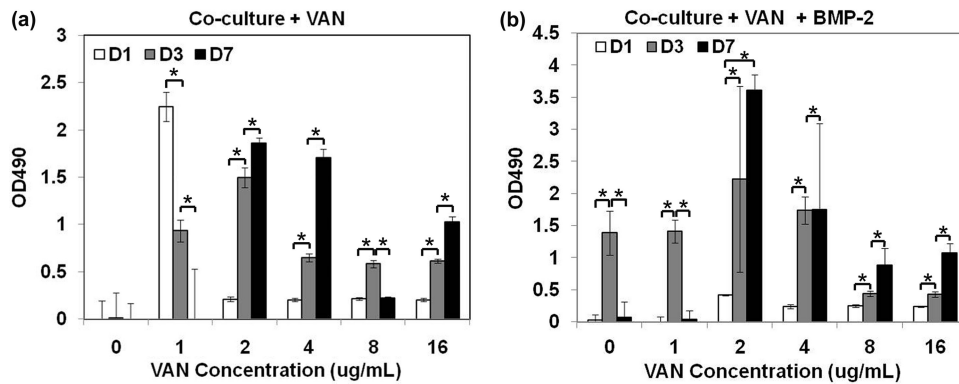


FIG 6 Measure of metabolic activity of W-20-17 in coculture treated with 0 to 16 µg/mL VAN in combination with (a) 0 ng/mL BMP-2 or (b) 100 ng/mL BMP-2 over 7 days using an MTS colorimetric assay. Higher OD₄₉₀ values indicate greater metabolic activity. The initial cell density was 15,000 cells per well. The absorbance is expressed as a measure of the cell viability via cell culture media for 7 days of incubation. Each value represents the mean \pm SD ($n = 3$). *, statistically significant difference between different time points ($P < 0.05$).

W-20-17 cells per capita, we expect that cocultures with high bacterial concentrations would show low ALP-specific activities whereas those with high numbers of W-20-17 would show values closer to that of the W-20-17 monoculture.

IL-6 production of cells in coculture. Interleukin-6 (IL-6) is a protein found to be secreted by human and mouse osteoblasts at elevated levels in response to infection by bacteria (specifically *S. aureus*) (6). Our results indicate that IL-6 production was significantly increased only upon exposure to bacteria in groups with VAN treatment at 16 µg/mL (Fig. 9). VAN treatments at ≤ 1 µg/mL resulted in a reduction in the amount of IL-6 produced. This is likely attributable to a decrease in the raw numbers of W-20-17 cells in culture. Treatments with VAN at 2 to 8 µg/mL showed no

significant change in IL-6 production. Groups treated with VAN at 16 µg/mL showed an increase in the amount of IL-6 produced. This suggests a greater number of cells combating infection by *S. aureus*.

DISCUSSION

The goals of this study were to establish an *in vitro* coculture model system to better represent the *in vivo* scenario of dual drug delivery and to gain a better understanding of the interactions between two specific compounds of choice: vancomycin antibiotic and bone morphogenetic protein 2. Briefly, we treated mouse bone marrow stromal cells (W-20-17) and *S. aureus* cells (ATCC 6538) with various concentrations of VAN (0 to 16 µg/mL) and BMP-2 (0 or 100 ng/mL) in both mono- and cocultures. In order to assess cell proliferation, differentiation, morphology, and inflammation response, an array of assays were performed for each treatment group across cultures grown for periods ranging from 1 h to 7 days. We selected the W-20-17 cell line because it has been used for an ASTM (F2131) standard test for *in vitro* biological activity of BMP-2. *S. aureus* was chosen for both its clinical origin and its high prevalence in bone graft failures (2, 4, 19, 29). If desired, modification of the system we establish here could accommodate testing of other antibiotic or growth factor compounds across a wide array of mammalian/bacterial cell cocultures. Similarly, the utilization of other antimicrobials such as silver ions should also be considered in order to complement the activities of substances such as VAN and BMP-2 (42, 49).

With regard to the efficacy of VAN antibiotic, we found that in coculture, VAN behaved differently than expected based on our monoculture findings. Staining results showed that in cocultures with BMP-2 at both 0 and 100 ng/mL, 2 µg/mL VAN was effective for 1 day, 4 µg/mL for 3 days, and 8 µg/mL for 7 days. This suggests that while bacteria were kept in check by some concentrations of VAN early on, concentrations below 8 µg/mL were not effective at reducing bacterial growth for extended periods of time. Compared to *S. aureus* monoculture L/D staining, coculture MTS results showed that in coculture, a higher dosage of VAN (8 µg/mL versus 2 µg/mL) was necessary to effectively inhibit bacterial growth for the same treatment group in monoculture (Fig. 5 and 6). By day 7, it would be expected that the *S. aureus* monocultures treated with at least 2 µg/mL VAN would continue to show little to

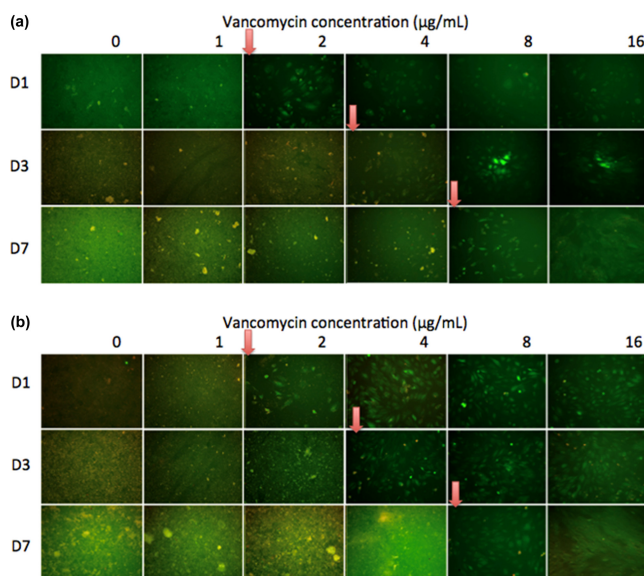


FIG 7 Live/Dead fluorescence staining of *S. aureus* and W-20-17 coculture treated with 0 to 16 µg/mL VAN in combination with (a) 0 ng/mL BMP-2 or (b) 100 ng/mL BMP-2 in DMEM-p medium. Photos were taken at $\times 100$ magnification after 1, 3, and 7 days of culture. Green indicates live cells, whereas red indicates dead cells. Smaller, spherical shapes are indicative of bacteria, while larger, more irregular shapes are W-12-17 cells. Red arrows indicate effective dosage of VAN.

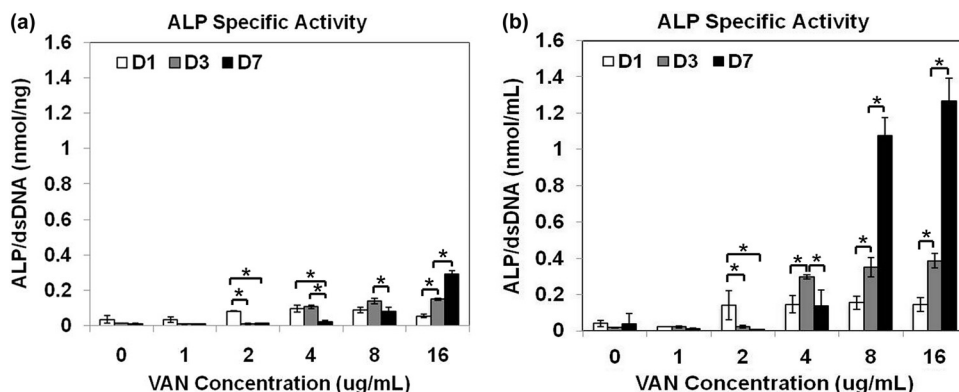


FIG 8 Measure of early osteoblastic differentiation of W-20-17/*S. aureus* coculture treated with 0 to 16 µg/ml VAN in combination with (a) 0 ng/ml BMP-2 or (b) 100 ng/ml BMP-2 using an ALP assay. Measurements were taken after 1, 3, and 7 days in culture and normalized for the dsDNA content. ALP activity is expressed as nanomoles per nanogram. Each value represents the mean \pm SD ($n = 3$). *, statistically significant difference between different time points ($P < 0.05$). VAN treatments had a significant effect on ALP-specific activity. Higher concentrations of VAN were correlated with higher ALP-specific activities, suggesting that most of the activity was being generated by mBMSCs in culture, as opposed to *S. aureus*, which showed significantly lower ALP-specific activity (see Fig. 7). Treatment with 100 ng/ml BMP-2 yielded significantly higher ALP-specific activity than treatment without BMP-2. These results suggest that in coculture, both VAN and BMP-2 are able to maintain their respective functions.

no bacterial growth, but the accuracy of this approximation requires further testing. Still, this suggests that monocultures may not be a representative model for antibiotic dose dependence in our coculture model. Furthermore, this suggests that the coculture environment may limit the efficacy of VAN. It has been proposed that internalization of the *S. aureus* by the osteoblasts allows the bacteria to evade any vancomycin in solution, thus reducing the efficacy of the drug (13). However, further investigation is required to elucidate this mechanism, either through mechanistic study or through testing of alternative cell lines and compounds.

In both mono- and cocultures, we found that for all concentrations tested (0 to 16 µg/ml), VAN had no evident effect on the growth of mouse bone cells. Even at VAN concentrations of up to 200 µg/ml (data not shown), we found that the antibiotic had no detrimental effect on W-20-17 proliferation, differentiation, or morphology. This result holds true for both for the cultures that received only VAN and the cultures that also received BMP-2. Clearly, VAN is a good candidate as the antibiotic of choice in our

proposed dual-delivery system in that it is effective against *S. aureus*, the primary offender in bone graft failure, and has no evident toxicity to host osteoblasts at effective concentrations. Furthermore, our results are consistent with the available literature (35).

The second prong of our proposed dual-delivery system, BMP-2, showed that it is capable of creating a significant increase in ALP production (an early marker of osteoblastic differentiation) in both mono- and cocultures. In our qualitative L/D staining assessment, cultures treated with BMP-2 at 100 ng/ml showed better growth than those without BMP-2 treatment. This qualitative result is backed by the quantitative MTS and ALP assay results. ALP-specific activity was used to differentiate between bacterial proliferation (low ALP-specific activity) and mBMSC proliferation (high ALP-specific activity). On average, treatment with BMP-2 resulted in a statistically significant increase in ALP-specific activity (from less than 0.5 nmol/ml to as much as 2.5 nmol/ml). This pattern is also evident in coculture, suggesting that BMP-2 maintains its functionality in both mono- and cocultures. BMP-2 showed little to no effect both qualitatively and quantitatively on the monocultures of ATCC 6538 *S. aureus* within the 24-h time frame tested. This remained true for all concentrations of VAN tested, further positing that BMP-2 can maintain its positive effect on mouse bone cells while generating little to no positive effect on bacterial cells.

While BMP-2 and VAN behaved as expected when delivered in coculture, their combined effects on both the W-20-17 and *S. aureus* have, to our knowledge, never been established before despite their dual delivery previously having been explored (18, 27, 45). It is important to emphasize that across all concentrations of VAN tested, BMP-2 had no measurable detrimental effect on the drug's action against the Gram-positive bacteria. In fact, we found that some assays indicated that in treatments with BMP-2, a lower concentration of vancomycin was able to achieve almost complete suppression of bacterial growth compared to treatments without BMP-2 (VAN at 8 µg/ml in cocultures with BMP-2 versus 16 µg/ml in cocultures without BMP-2). The true mechanism behind this result cannot be determined due to the limitations of our assays, but we believe that it may have been a result of

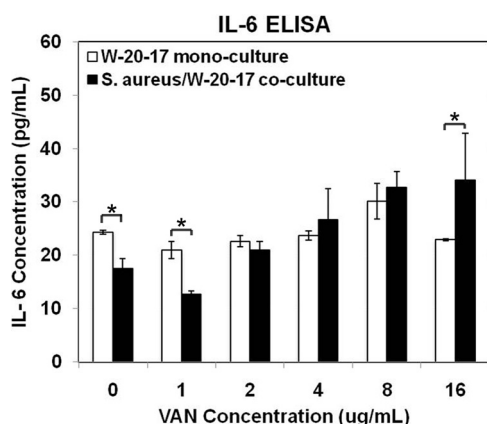


FIG 9 Measurement of W-20-17 inflammation response in coculture with *S. aureus* and treated with 0 to 16 µg/ml VAN and 100 ng/ml BMP-2 using an IL-6 ELISA kit. *, statistically significant difference between monoculture and coculture ($P < 0.05$).

BMP-2 conferring a competitive advantage upon the mBMSCs over the bacteria. It is suggested that BMP-2 allows improved cell growth and differentiation by day 7, thus allowing bone cell growth to outpace the capacity of the bacteria to infect and proliferate. Because this effect has not been observed before, further investigation into this interaction is necessary. If found to be true, this type of interaction would be suspected to be observed in treatments with other combinations of antibiotics and growth factors capable of maintaining function when delivered together. Such a finding could very well bring the study of prohost/antimicrobial compound dual delivery to the forefront of anti-infection research.

Here we begin an attempt to bridge the two major avenues of study in bone tissue repair: accelerated growth and improved treatment success rates. While each of these concepts has already been implemented in standard clinical care in the form of antibiotic and bone growth factor delivery, there still remains a need for refinement in their execution. This is the first of many studies aimed at creating a streamlined dual-delivery system that is capable of delivering both antibiotic drugs and bone growth factors to achieve those aforementioned goals. We have established an mBMSC/*S. aureus* coculture model system that is aimed at mimicking the *in vivo* conditions of bone graft infection and giving consistent, predictable results. From this model system, we have begun to characterize the interactions of two drugs of interest: VAN antibiotic and BMP-2 growth factor. Our findings indicate that, individually, they maintain their expected functionality as an antibiotic and growth factor, respectively, but that, in combination, their combined effects may result in a lower demand for the use of toxic, expensive VAN. As a whole, these results pave the way for future study in the dual delivery of antibiotics and growth factors. We plan to begin testing both delivery timing and more material-focused studies regarding the actual mechanistic approach to delivering said components using the coculture model system established here.

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